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PRINCIPAL INVESTIGATOR: Isaac Cohen, Ph.D.

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13. ABSTRACT *(Maximum 200*

The overall objective of this research proposal is to improve the capacity of stem cells to engraft the megakaryocytic lineage. *Ex vivo* expansion of bone marrow megakaryocyte progenitors in the presence of thrombopoietin (TPO) was significantly enhanced by a soluble factor present in cultures of confluent marrow stromal cells. We are presently attempting to identify this factor. Addition of interleukin-3, but not stem cell factor, to TPO, enhances the expansion of peripheral blood megakaryocyte progenitors from CD34+ cultures, similarly to bone marrow cells. The evaluation of megakaryocytopoiesis from PIXY321 vs. GM-CSF-mobilized peripheral blood progenitor cells yielded the following results: the expansion efficiency of megakaryocytes (MK), defined as the number of MK produced per seeded CD34+ cells, was greater in PIXY321- than in GM-CSF-mobilized samples. However, since the frequency of CD34+ cells was greater in GM-CSF- than in PIXY321-mobilized samples, there was no significant difference in the overall absolute number of MK produced in PIXY321- and GM-CSF-mobilized samples. These results will be useful for *ex vivo* expansion of MK to treat post-transplant thrombocytopenia.

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Isaac Cohen 9/26/96

PI - Signature Date

Table of Contents

INTRODUCTION

Following high-dose chemotherapy for the treatment of malignant disease, reinfusion of peripheral blood stem cells collected during treatment with growth factors usually results in delayed platelet engraftment as compared to neutrophil recovery (1-4). The overall objective of this research proposal is to improve the capacity of stem cells to engraft the megakaryocytic lineage. To achieve this goal we used mobilized peripheral blood progenitor cells (PBPC) from patients enrolled in the clinical trial NCI #T93-0058 and randomized to receive either rGM-CSF or the new recombinant growth factor PIXY321. We compared these PBPC to bone marrow (BM) and fetal cord blood (FCB) cells in their capacity to undergo megakaryocytopoiesis.

As mentioned in progress report 01, we have substituted thrombopoietin, the purified growth factor mainly specific for the MK lineage, for the aplastic serum mentioned in the original proposal.

BODY OF PROGRESS REPORT

Materials and Methods:

Preparation of low density non-adherent mononuclear cells (MNCs): BM, FCB and MPBSC samples were collected in accordance with the guidelines of the Institutional Review Board on Human Subjects. BM, obtained from the femur of hematologically normal patients having total hip arthroplasty, was collected in a special anticoagulant mixture designed to prevent platelet activation and containing final concentrations of 50 U/ml preservative-free heparin, 1 mM Na_2EDTA, 1 mM adenosine, 2 mM theophylline, 2.2 µM prostaglandin E_1 and 0.1 mg/ml DNase I. Marrow cells were repeatedly extracted from bone fragments with a modified MK medium (5) which consists of Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (Dulbecco's PBS, Gibco) containing 13.6 mM Na citrate, 11 mM dextrose, 1 mM theophylline, 1 % bovine serum albumin, 2.2 µM PGE_1 and 0.1 mg/ml DNase I. Following homogenization by passage through a 18 gauge needle, low density cells were extracted with the use of Ficoll-Paque as described (6). Cells resuspended in MK medium were centrifuged at 380xg through a 10 % human serum albumin cushion in PBS to reduce platelet contamination. Residual red cells were lyzed with NH_4Cl as described (7) and the remaining cells recovered by centrifugation through a 10 % human serum albumin cushion. Adherent cells were discarded following overnight incubation in Iscove Modified Dulbecco's medium (IMDM) containing 10 % fetal bovine serum (FBS). All culture media were supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 4 µg/ml gentamycin, and incubation carried out at 37°C in a 5 % CO_2 fully humidified atmosphere.

FCB was collected in the special anticoagulant. Buffy coat and red cells were submitted to Ficoll separation and the remaining steps carried out as for bone marrow cells. Frozen MPBSC were rapidly thawed at 37°C and added to thawing medium consisting of IMDM, 25% FBS, 12.5 U/ml heparin and 1.25 mg% DNase I. Residual red cells were lyzed, and remaining MNCs were washed and treated as described above for the elimination of adherent cells.

Purification of CD34+ cells: CD34+ cells were purified by positive selection using the CD34 magnetic cell sorting Mini-MACS kit (Myltenyi Biotec, Auburn, CA) in accordance with the manufacturer's recommendation. A recovery of about 60 % CD34+ cells was obtained with a purity of 86.4 % ± 1.5 S.E.M (n=6) on the basis of flow cytometric analysis following staining with PE-anti-CD34 (HPCA-2).

Culture conditions: Low density non-adherent mononuclear cells (MNC) and purified CD34+ cells were cultured for 12-14 days at 37°C at concentrations of 10^6 and $5x10^4$ cells/ml, respectively, in an IMDM-based medium with 1 % human serum albumin and 2.5 % normal human serum which was found to be necessary for MK cultures. In order to prevent the inhibitory effects on MK growth of transforming growth factor-ß, ß-thromboglobulin and platelet factor 4 released from activated platelets (8-10), normal serum was obtained by recalcification of citrated platelet-free

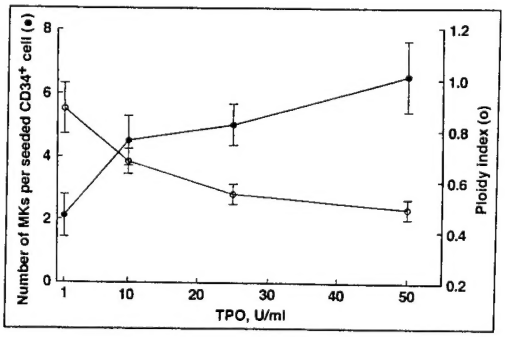
plasma. TPO (Zymogenetics Corp.) was used at a concentration of 50 U/ml (10 U = TPO quantity which stimulates one-half maximal proliferation of BaF3/mpl cells) which yielded the maximal concentration of MKs. Concentrations of IL-3 and Stem Cell Factor (SCF) (R&D Systems, Minneapolis, MN), were 10 ng/ml (0.4-1.6 units based on 1 unit=ED50 of TF1 cell line proliferation) and 50 ng/ml, respectively, and were optimal for MK growth.

Cell labeling and flow cytometric analysis: The relative frequency of MK progenitors was determined at day 1 on MNC and CD34+ cells. Cell aliquots were treated with 200 pkat chymopapain (Knoll Pharm. Co., Lincolnshire, IL) This treatment detaches most platelets and platelet fragments from cells which otherwise would stain with the anti-CD41a antibody (anti-GPIIb/IIIa). After washing the cells were double-stained with phycoerythrin (PE) conjugated-anti-CD34 (HPCA-2, Becton-Dickinson) and fluorescein isothiocyanate (FITC) conjugated anti-CD41a (Immunotech-Amac, Westbrook, ME) and analyzed by flow cytometry. Negative controls were PE-anti-mouse IgG_1 and FITC-anti-mouse IgG_1 used at equivalent IgG_1 concentrations. The relative frequency of mature MKs was determined following 12-14 days culture by flow cytometric analysis of cells stained with FITC-anti-CD41a. Flow cytometric analysis was performed using a Coulter ELITE dual laser flow cytometer. Fluorescence attributable to FITC- and PE-labeled antibodies was determined using excitation by an argon laser operating at 488 nm and adjusted to 0.3 W. Emission from fluorescein and PE was measured using band pass filters of 530 ± 15 nm and 575 ± 15 nm, respectively. The per cent positive cells was calculated by substracting the percent positive of the isotype control within the same integration region.

Statistical analysis: The mean number of CD41a+ cells per seeded CD34+ cells and the mean ploidy index were compared across study conditions using two factor analysis of variance (ANOVA) with sample and study condition as the two factors. If the ANOVA p-value was less than 0.05, then pairwise comparisons were done between each pair of groups using the paired t-test with statistical significance indicated when $p < 0.05$. Separate analyses were done for mononuclear cells and for CD34+ cells. Stroma-free and stroma non-contact conditions were compared using the paired t-test. PB MNC comparisons were done using the two-tailed Mann-Whitney U rank sum test.

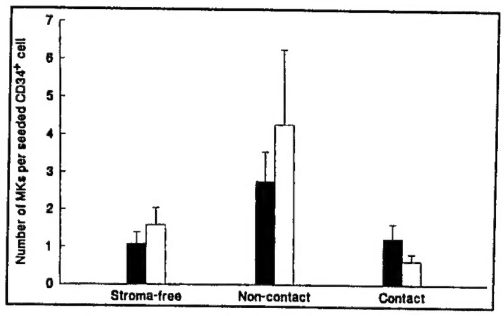
Results and Discussion:

Ex vivo expansion of BM and regulation by stroma: In our year 1 report, we had described the enhancing effect of IL-3 on TPO-stimulated megakaryocytopoiesis while SCF did not exhibit such effect. We now report that while TPO induces MK proliferation in a concentration-dependent manner, MK ploidy was inversely related to TPO concentration (Fig. 1). Furthermore, TPO-stimulated megakaryocytopoiesis in cultures of CD34+ cells, carried out in transwells, was enhanced by BM stroma present in wells (Fig. 2). We are now in the process of identifying this stroma-enhancing factor. We published these results in Stem Cells (11, see Appendix).



(●) proliferation expressed by absolute number of MKs/seeded CD34⁺ cells; (○) ploidy index (%4N + %8N + %16N + %32N/%2N). Inverse relationship is observed between proliferation and ploidy. Each value represents the mean ± SE of seven to nine separate experiments. p < 0.005 for 1 and 10 U/ml of TPO versus 50 U/ml TPO for both MK/CD34 and ploidy.

Fig. 1. Effect of different TPO concentrations on MK proliferation and ploidy in MNC cultures.



Black bars, human stroma; Empty bars, M2-10B4 murine stroma cell line. Mean (± SE) number of CD41a+ cells per seeded CD34+ cell by study condition. For human stroma, p = 0.04 for "stroma-free" versus "noncontact."

Fig.2. Effect of stroma on TPO-stimulated CD34+ cells.

Ex vivo expansion of PBPCs: MNCs from 18 patients enrolled on the PIXY321 study were cultured at 1 million cells/ml with the standard cytokine panel. The cultures were analyzed at Day 12 for %CD41+ cells. No significant differences were seen by adding any combination of cytokines.

CD34 cells from seven peripheral blood stem cell phereses were selected out. An average 65.4% purity was obtained. 50,000 cells/ml were cultured with the standard cytokine panel. The cultures were analyzed at Day 12 for %CD41+ cells (Table 1). Adding 10 ng/ml IL-3 significantly increased the number of MKs produced ($p = 0.016$). Adding 100 Ng/ml SCF increased the MK yield, but not significantly ($p = 0.16$). IL-3 and SCF together also significantly increased the number of MKs produced ($p = 0.009$), however SCF did not significantly increase MK yield versus adding IL-3 alone ($p = 0.89$). In conclusion, adding IL-3 to CD34 cells grown in the presence of TPO will increase MK yield. These results are similar to those reported for BM in year 01 (see reprint of paper in Appendix).

Table 1.

Yield of MK/seeded CD34+ cells ± SEM

TPO	TPO + IL-3	TPO + SCF	TPO + IL-3 & SCF
3.04 ± 1.0	8.19 ± 2.7	5.50 ± 1.2	9.30 ± 3.2

Comparison of MK yield from BM, FC and PB MNC and CD34+ cells : While carrying cultures for 36 days, we noticed that the greatest yield of MKs was obtained at day 18. Due to the large variability between samples we need to generate more results to achieve statistical significance. FC, however, appears to be a more productive source of MKs than either BM or PBPC.

In vitro production of megakaryocytes from PIXY321 vs. GM-CSF-mobilized peripheral blood progenitor cells: Table 2 shows the clinical features of patients whose PBPCs were mobilized with either GM-CSF or PIXY321.

Table 2.

Cytokine used		PIXY321	GM-CSF
Number of Patients		10	8
Age (Median)		44 (Range: 33-57)	39 (Range: 30-41)
Bone Marrow Involvement		10%	12.5%
Prior Adjuvant Chemotherapy		30%	75%
Prior Radiation Therapy			
	Chest Wall	10%	25%
	Other	0%	12.5%
Sites of Disease			
	Bone	40%	63%
	Lung	10%	38%
	Lymph Node	40%	12.5%
	Bone Marrow	10%	12.5%
	Chest Wall	10%	38%

When PBPCs were mobilized with cytokine alone, GM-CSF patients had a significantly higher percentage of CD34+ cells in the non-adherent MNC population compared to PIXY321 patients (GM-CSF, 0.35% ± 0.09 vs. PIXY321, 0.10% ± 0.014, p = 0.006). In apheresis products collected during cytokine-stimulated recovery from CY treatment, the mean percentage of CD34+ cells in GM-CSF-mobilized MNC was three times higher than the mean for PIXY321-mobilized MNC, but did not reach statistical significance due to high variance (CY followed by GM-CSF, 1.61% ± 0.56 vs. CY followed by PIXY321, 0.56% ± 0.15, p = 0.08). Mobilization with myelosuppressive chemotherapy (CY) followed by cytokine produced a significantly higher percentage of CD34+ cells in the non-adherent MNC population in both patient groups (PIXY321 vs. CY followed by PIXY321, p = 0.006; GM-CSF vs. CY followed by GM-CSF, p = 0.008).

There were no significant differences between the MK/MNC yields of GM-CSF and PIXY321-mobilized patient samples, either when mobilized with cytokine alone (GM-CSF, 0.025 ± 0.007 vs. PIXY321, 0.017 ± 0.003, p = 0.56) or CY followed by cytokine (CY followed by GM-CSF, 0.197 ± 0.149 vs. CY followed by PIXY321, 0.064 ± 0.018, p = 0.61). MNCs collected during cytokine-stimulated recovery from CY produced significantly more MK/MNC than cytokine mobilization alone in every patient tested (PIXY321 vs. CY followed by PIXY321, p = 0.002; GM-CSF vs. CY followed by GM-CSF, p = 0.016).

The number of MK/MNC recovered at day 12 correlated directly with the %CD34+ in the MNC at day 1 for both PIXY321 and GM-CSF-treated patients, both when mobilized with cytokine alone or CY followed by cytokine (Figure 1). This was true whether the two groups were analyzed separately (PIXY321 r = 0.69, p<0.001; GM-CSF r = 0.91, p<0.001), or combined (Figure 3).

The yield of MK per CD34+ cell seeded was calculated. There was no statistical difference in the yield of MK/CD34+ between PBPCs mobilized with cytokine alone and CY followed by cytokine (PIXY321, 23.0 ± 7.8 vs. CY followed by PIXY321, 20.6 ± 8.3, p = 0.32; GM-CSF, 6.3 ± 2.8 vs. CY followed by GM-CSF, 7.8 ± 2.7, p = 0.99). When the results for mobilization with cytokine alone and CY followed by cytokine were combined, PIXY321-mobilized PBPCs had a higher mean MK/CD34+ cell seeded than GM-CSF-mobilized PBPCs (PIXY321 ± CY, 21.8 ± 5.53 vs. GM-CSF ± CY, 8.37 ± 1.92, p = 0.037).

A paper describing these results has been submitted for publication.

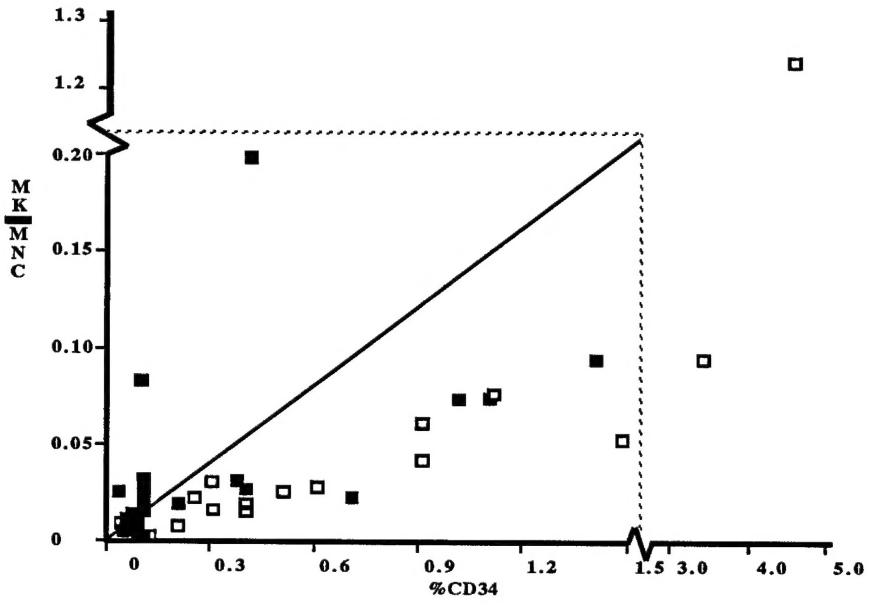


Figure 3. *Correlation of MK/MNC vs. %CD34+.*

The number megakaryocytes at day 12 per seeded non-adherent mononuclear cell on day 1 was correlated with the %CD34+ cell population in the non-adherent mononuclear cell on day 1 using the Spearman's rank analysis to take into account outlying data points. All PIXY321 (■) and GM-CSF ( ) samples were combined. Graph was extended to show outliers.r = 0.70, $p < 0.001$.

CONCLUSIONS

The following new results have been obtained in year 02:

1) We have further elucidated megakaryocytopoiesis in bone marrow and reported an enhancing effect of an as yet unidentified factor secreted by bone marrow stroma. These results were published in Stem Cells (11, see Appendix).

2) In year 01, we had reported that the small concentration of IL-3 used (0.16 ng/ml) may explain its lack of enhancement of TPO-induced megakaryocytopoiesis in PBPC. This was confirmed, and table 1 shows a statistically significant enhancing effect of IL-3 in cultures of CD34+ cells when used at a concentration of 10 ng/ml.

3) Megakaryocytopoiesis from PIXY321 vs. GM-CSF- mobilized PBPC was evaluated. The expansion efficiency of MK, defined as the number of MK produced per seeded CD34+ cell, was greater in PIXY321-mobilized samples than in GM-CSF-mobilized samples. However, the frequency of CD34+ cells was greater in GM-CSF-mobilized samples than in PIXY321 mobilized samples. Overall, there was no significant difference in the absolute number of MK produced per mononucleated cell between PIXY321 and GM-CSF-mobilized samples. These results point to the potential influence of the cytokine (s) used for mobilization on the outcome of *ex vivo* expansion. We have submitted these findings for publication.

A few weeks ago, we notified your Research Management that the clinical trial, NCI #T93-0058, "A randomized phase III comparison of PIXY321 and GM-CSF for mobilization of hematopoietic progenitors in metastatic breast cancer" has been closed to further accrual as of May 1, because the Immunex Corporation has decided to cease development of PIXY321 and to close all related clinical trials. We have therefore decided to use any apheresis product obtained from patients with breast cancer mobilized with cytokines at Northwestern Memorial Hospital and to compare their capacity to generate megakaryocytes (MK) to BM and FCB cells (for further clarification see letters of Dr. Jane Winter, co-investigator, in Appendix).

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APPENDIX

1. Publication in Stem Cells.

2. Copies of Letters from Dr. Jane Winter, co-investigator, clarifying the closure of PIXY321 clinical trials, and Revised Statement of Work.

3. New Consent Form for the use of a sample of PB, normally harvested for stem cell transplant, for *in vitro* study. Human Subject IRB approval forthcoming.

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August 14, 1996

Ref. Grant No. DAMD17- 94 - J - 4465

Dear Ms. Hackley:

In reference to your letter of July 2, 1996, Dr. Jane Winter, Co-Investigator in charge of the clinical trial, is providing in the attached document clarifications pertaining to the changes discussed in her letter of May 15, 1996. Enclosed please also find a revised Statement of Work. We will send you the copy of approval from the IRB for the new protocol and consent form as soon as we get it.

Sincerely yours,

Isaac Cohen, Ph.D.

Ms. Margo Lucareli
Associate Director
Office of Research and
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c.c.: Ms. Catherine Smith, MCMR-RCQ-PLF

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Jane N. Winter, MD
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August 8, 1996

Ms. Kathy Hackley
Contract Specialist
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Re: Grant No. DAMD17-94-J-4465

Dear Ms. Hackley:

I am writing to update you regarding the status of NCI Protocol #T93-0058, A Randomized Phase III Comparison of PIXY321 and GM-CSF for Mobilization of Hematopoietic Progenitors in Metastatic Breast Cancer." On May 15, I wrote to inform you that the clinical trial was closed to further accrual on May 1, because the Immunex Corporation has decided to cease development of PIXY321 and to close all related clinical trials. It was our intention to revise the study as a Phase II clinical trial using commercially available GM-CSF, and that it would remain active at Northwestern Memorial Hospital.

Whereas the primary objectives of the original phase III study had been achieved, the revised protocol was to address the secondary objectives - i.e. to "assess the antitumor response, survival and disease-free survival following dose-intensive chemotherapy followed by autologous stem cell rescue and consolidation radiation therapy to sites of pre-treatment bulk disease." The protocol had been completely reformated and revised accordingly. To prepare the statistics section for the revised protocol and to determine the projected accrual, we updated our response and survival data for all patients who were sufficiently out from their transplant to be evaluable. Using the American Bone Marrow Transplant Registry's recently updated data, we compared our results on the randomized trial to the existing compilation of data for the North American patients with similar stage disease. With our statistician, Dr. A. Rademaker, we determined that our results were so similar to the existing data, that it would take the accrual of an unreasonably large number of patients to demonstrate the superiority of the more intensive approach taken in this protocol. In a meeting of the Robert H. Lurie Cancer Center's Protocol Review Committee,

yesterday, there was unanimous agreement that it was a mistake to pursue the secondary objectives given the large number of additional cases required to prove a difference.

A new consent to permit study of any apheresis product obtained from patients with breast cancer mobilized with cytokines at Northwestern Memorial Hospital will be submitted to the IRB. As required by the grant, these specimens will be studied and compared to bone marrow and umbilical cord blood for their capacity to generate megakaryocytes <u>in vitro</u>.

Our data manager will continue to collect data on the many patients who have undergone transplant. The last patient to be entered on trial has only recently been discharged from the hospital. Engraftment will be tracked, as well as response, as dictated by the study.

A revised "Statement of Work" is attached. If further information is required, please contact Dr. Isaac Cohen directly.

Sincerely yours,

Jane N. Winter, M.D.
Director, Bone Marrow Transplant
Robert H. Lurie Cancer Center of
Northwestern University

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May 15, 1996

Ms. Kathy Hackley
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Grant No. DAMD17-94-J-4465
Project Title: Megakaryocytopoiesis in Stem Cell Transplantation
Principal Investigator: Isaac Cohen, Ph.D.

Dear Ms. Hackley:

I am writing to notify you that the clinical trial, NCI #T93-0058, "A Randomized Phase III Comparison of PIXY321 and GM-CSF for Mobilization of Hematopoietic Progenitors in Metastatic Breast Cancer" has been closed to further accrual as of May 1, because the Immunex Corporation has decided to cease development of PIXY321 and to close all related clinical trials. Patients currently in process will continue to be treated on-study, and all previously entered patients will be followed for the planned three-year interval. Data collection will continue as required.

This clinical trial will be revised as a phase II study using commercially available GM-CSF, and will remain active at Northwestern Memorial Hospital. It will **not** be administered through the NCI/CTEP. Samples from the aphereses performed on this trial will be studied as previously described.

In addition, a successor study is under development and study of the aphereses for megakaryocyte growth and differentiation as per DAMD17-o4J-4465 will occur. When the full protocol and consent become available, they will be forwarded to you.

Sincerely yours,

Jane Winter, M.D.

Jane N. Winter, M.D.
Associate Professor of Medicine
Director, Bone Marrow Transplant Program
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Isaac Cohen

Isaac Cohen, Ph.D.
Professor, Department of Cell, Molecular and Structural Biology
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APPENDIX G. REVISED STATEMENT OF WORK - Grant No. DAMD17-94-J-4465

Three distinct sources of hematopoietic progenitor cells will be compared for their capacities to undergo megakaryocytopoiesis. In the previous Statement of Work, we proposed that the samples which will be processed comprise 40 mobilized peripheral stem cells (MPBSC) from patients randomized to receive either GM-CSF or PIXY321 and twenty each bone marrow (BM) and fetal cord blood (FC) samples or a total of 80 samples. We propose a change in the clinical trial since Immunex Corp. ceased production of PIXY321. MPBSC will be collected from patients randomized to receive either Synthokine/G-CSF or G-CSF or GM-CSF. Mononuclear cells and CD34+ cells will be isolated from each hematopoietic source and cultured in the absence or presence of cytokines. CD34+ cells will additionally be cultured in inserts separated from stromal cells by a membrane. These cultures will be carried out in the presence and absence of purified cytokines.

Since each sample will be submitted to six different culture conditions, a total of 480 cultures will be carried out.

By August 14, 1996, 18 samples from patients mobilized with PIXY321 or GM-CSF have already been studied as well as 10 BM and FC samples.

Year 03: Processing 12 MPBSC from patients mobilized with Synthokine/G-CSF or G-CSF or GM-CSF as well as 6 BM and 6 FC samples.

Year 04: Processing the remaining 10 MPBSC and 4 each BM and FC. Statistics to be performed and correlation of results obtained with MPBSC with time to platelet engraftment post-stem cell transplant to be carried out.

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September 17, 1996

To: Joanne Richmond

From: Jane N. Winter, M.D.

I am co-investigator on two funded laboratory research projects that utilize peripheral blood progenitor cells. The two clinical trials which provided these specimens have now closed (NU93B1 and NU93H3). The first was closed because the company decided not to develop PIXY321 and terminated all clinical trials. The second study met accrual.

Therefore, I am submitting the attached consent form which will provide specimens for either research study. Whereas Dr. Cohen's Megakaryocytopoiesis grant is now due for its annual renewal, I am submitting this consent with it. This consent will also be used to provide specimens for Dr. William Miller's study, "Bioreactor Design for Ex Vivo Expansion of Hematopoietic Cells," which is funded by the National Science Foundation.

I hope this will simplify things.

nnn

EX VIVO EXPANSION STUDIES:

(a)MEGAKARYOCYTKOPOIESIS IN STEM CELL TRANSPLANTATION
Project Directors: Drs. Isaac Cohen and Jane Winter

(b) BIOREACTOR DESIGN FOR EX VIVO EXPANSION OF HEMATOPOIETIC CELLS
Project Directors: Drs. William Miller and Jane Winter

I have been invited to participate in a study designed to develop methods to grow blood stem cells in the laboratory. I have already signed consent to undergo collection of parent blood cells from the blood circulating in my veins (apheresis). I willingly consent to the use of one-half of one teaspoonful of each collection for research purposes.

The purpose of this research study is to study the effects of different "growth factors" on stem cells in the laboratory. Growth factors are proteins that stimulate immature blood cells to grow. It is the goal of this study, to determine the best combination of growth factors and conditions to grow blood stem cells. This research study may provide the information required to expand the number of blood stem cells collected in a single apheresis many-fold in the future. This would reduce the number of stem cell collections required for a stem cell transplant.

There may be no direct benefit to me at this time; however, the results of the study should further scientific knowledge in this area.

Donation of a portion of the apheresis collection will not involve any additional blood draws. The total number of cells contributed to the research project will represent only a small fraction of the collection, and will not present a risk to me.

My participation is voluntary and I am free to withdraw at any time without penalty or loss of benefits. Any questions I have will be answered by Dr. Jane Winter at 312-908-5400.

This research project is supported in part by the U.S. Army Medical Research and Development Command (USAMRDC). It is the policy of the USAMRDC that data sheets be completed on all volunteers participating in research for entry into the USAMRDC's Volunteer Registry Data Base. The information to be entered into the data base includes my name, address, social security number, the study name and date. The intent of the data base is two fold: first, to readily answer questions concerning an individual's participation in research sponsored by USAMRDC; and second, to ensure that the USAMRDC can exercise its obligation to ensure that research volunteers are adequately warned of risks and to provide new information as it becomes available. The information will be stored at USAMRDC for a minimum of 75 years. The computer data base will be kept confidential and not released to anyone.

I agree that representatives from the U.S. Army Medical Research, Development, Acquisition and Logistics Command are eligible to inspect the records of this research as part of their responsibilities to protect human subjects in research.

I have been notified that the peripheral blood stem cells that I am providing will not be used in another protocol.

Date	Patient's Signature
Date	Physician's Signature
Date	Witness's Signature

Thrombopoietin-Stimulated ex Vivo Expansion of Human Bone Marrow Megakaryocytes

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Key Words. Thrombopoietin • Megakaryocytopoiesis • Mononuclear cells • CD34+ cells • Bone marrow • Cytokines

Abstract. The effect of thrombopoietin (TPO) on megakaryocytopoiesis (MKP) has been mainly studied using clonogenic assays in murine systems. In this study, we evaluated MKP in liquid culture using human bone marrow cells. While interleukin 3 (IL-3) and stem cell factor (SCF) are potent activators of TPO-stimulated MKP in the murine system, only IL-3 exhibited synergistic activity with TPO in cultures of human bone marrow. The IL-3 effect on TPO-stimulated megakaryocyte (MK) proliferation, expressed as the absolute number of MKs per seeded CD34+ cell, was more pronounced with purified CD34+ cells (8 ± 1.6 SE versus 2.8 ± 0.7 SE in the presence and absence of IL-3, respectively) than with mononuclear cells (MNC) (16 ± 2.8 SE versus 11 ± 2.0 SE). This effect of IL-3 on TPO-stimulated MK proliferation was due to a general proliferation of all cell types since the relative frequency of MKs (32.1 ± 3 SE and 55.8 ± 3 SE in MNC and CD34+ cells, respectively) was not affected by IL-3. The effect of TPO alone, TPO + IL-3, TPO + SCF, and TPO + IL-3 + SCF on MK proliferation was examined in MNC and CD34+ cultures. Greater numbers of MK per seeded CD34+ were observed in MNC compared to CD34+ cultures under all conditions except when TPO was added with both IL-3 and SCF. The enhancing effect of MNC was also observed on MK ploidy in the presence of TPO and IL-3. While proliferation and ploidy increase with TPO concentration in the murine system, they are inversely related in the human system. A significant 2.5-fold enhancement of TPO-induced MK proliferation was observed when purified CD34+ cells were cultured in inserts separated from human bone marrow stroma, indicating that soluble stimulatory factors are released from the stroma. These observations will be useful for ex vivo expansion of MKs to treat post-transplant or chemotherapy-associated thrombocytopenia. *Stem Cells 1996;14:207-214*

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Introduction

The recently cloned thrombopoietin (TPO) is a megakaryocyte (MK) lineage-specific growth factor which acts at all levels of megakaryocytopoiesis and thrombopoiesis [1-5]. Its availability will be of considerable importance for the treatment of thrombocytopenias following irradiation and chemotherapy. In view of the slow platelet engraftment following transplant of hematopoietic progenitors [6-8], the use of ex vivo expanded MKs as a transplant supplement could reduce the time for platelet recovery. In murine systems it has been demonstrated that interleukin 3 (IL-3) and stem cell factor (SCF) strongly potentiate the effect of TPO on MK colony formation [9, 10]. In this study, we evaluated MK expansion in vitro in liquid cultures of human bone marrow mononuclear cells (MNC) or purified CD34+ cells supplemented with TPO alone or in combination with IL-3 and/or SCF. Since megakaryocytopoiesis is regulated by bone marrow stromal cells [11, 12], we also evaluated the effect of

human and murine stroma on TPO-treated $CD34^+$ cells.

Materials and Methods

Preparation of Low Density Nonadherent MNC

Bone marrow (BM) samples were collected in accordance with the guidelines of the Institutional Review Board on Human Subjects. BM, obtained from the femur of hematologically normal patients having total hip arthroplasty, was collected in a special anticoagulant mixture designed to prevent platelet activation and containing final concentrations of 50 U/ml preservative-free heparin, 1 mM Na_2EDTA, 1 mM adenosine, 2 mM theophylline, 2.2 μM prostaglandin E_1 and 0.1 mg/ml DNase I. Marrow cells were repeatedly extracted from bone fragments with a modified MK medium [13] which consists of Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (Dulbecco's PBS, GIBCO; Grand Island, NY) containing 13.6 mM Na citrate, 11 mM dextrose, 1 mM theophylline, 1% bovine serum albumin, 2.2 μM PGE_1 and 0.1 mg/ml DNase I. Following homogenization by passage through a 21 gauge needle, low density cells were extracted with the use of Ficoll-Paque as described [14]. Cells resuspended in MK medium were centrifuged at $380 \times g$ through a 10% human serum albumin (HSA) cushion in PBS to reduce platelet contamination. Residual red cells were lysed with NH_4Cl as described [15], and the remaining cells were recovered by centrifugation through a 10% HSA cushion. Adherent cells were discarded following overnight incubation in α-thioglycerol-free Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS). All culture media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin and incubation was carried out at 37°C in a 5% CO_2 fully humidified atmosphere.

Purification of $CD34^+$ Cells

$CD34^+$ cells were purified by positive selection using the CD34 magnetic cell sorting Mini-MACS kit (Myltenyi Biotec; Auburn, CA) in accordance with the manufacturer's recommendation.

BM Stroma

Human BM stroma was prepared according to *Koller et al.* [16]. Essentially, BM MNC were plated in either collagen-free or collagen-coated 6-well plates in McCoy's 5A medium which was supplemented with FBS, horse serum, amino acids, vitamins and hydrocortisone. At confluency, the plates were irradiated with a dose of 12 Gy from a ^{137}Cs source (Gammacell 40, Nordion International Inc.; Kanata, Ontario, Canada). The M2-10B4 murine stromal cell line was kindly provided by *Dr. Connie Eaves* (Terry Fox Laboratory; Vancouver, BC, Canada) and grown over collagen-coated plates. At confluency, the plates were irradiated at 80 Gy. Collagen-coated plates were from Collaborative Research (Becton Dickinson; San Jose, CA).

Culture Conditions

Low density nonadherent MNC and purified $CD34^+$ cells were cultured for 12-14 days at 37°C at concentrations of 10^6 and 6×10^4 cells/ml, respectively, in an IMDM-based medium with 1% HSA and 2.5% normal human serum which was found to be necessary for MK cultures. The concentration of MNC and purified $CD34^+$ cells seeded contained equivalent concentrations of $CD34^+$ cells. In order to prevent the inhibitory effects on MK growth of transforming growth factor-β, β-thromboglobulin and platelet factor 4 released from activated platelets [17-19], the serum was obtained by recalcification of citrated platelet-free plasma. TPO (Zymogenetics Corporation; Seattle, WA) was used at a concentration of 50 U/ml (10 U = TPO quantity which stimulates one-half maximal proliferation of BaF3/mpl cells) which yielded the maximal concentration of MKs. Concentrations of IL-3 and SCF (R&D Systems; Minneapolis, MN), were 0.16 ng/ml (0.4-1.6 units based on one unit = ED50 of TF1 cell line proliferation) and 50 ng/ml, respectively.

The effect of stroma from either human BM or M2-10B4 murine cell line was evaluated by culturing purified $CD34^+$ cells in the presence of TPO (100 U/ml) either in direct contact with stroma (contact) or in inserts (Becton Dickinson; Franklin Lakes, NJ) separated from stroma by a 0.4 μm pore membrane (noncontact). In control experiments $CD34^+$ cells were cultured in inserts placed over stroma-free wells (stroma-free).

In all cultures, MK proliferation was expressed by the absolute number of $CD41a^+$ cells obtained per seeded $CD34^+$ cell. The latter was calculated from the relative frequency of $CD34^+$ cells determined in MNC and purified $CD34^+$ cells.

Cell Labeling and Flow Cytometric Analysis

The relative frequency of MK progenitors was determined at day 1 on MNC and purified $CD34^+$ cells. Cell aliquots were treated with 200 pkat chymopapain (Knoll Pharmaceutical Co.; Lincolnshire, IL). This treatment detaches most platelets and platelet fragments from cells which otherwise would stain with the anti-CD41a antibody (anti-GPIIb/IIIa) [20]. After washing, the cells were double-stained with phycoerythrin (PE) conjugated-anti-CD34 (HPCA-2, Becton Dickinson) and fluorescein isothiocyanate (FITC) conjugated anti-CD41a (Immunotech-Amac; Westbrook, ME) and analyzed by flow cytometry. Negative controls were PE-anti-mouse IgG_1 and FITC-anti-mouse IgG_1 used at equivalent IgG_1 concentrations. The relative frequency of mature MKs was determined following a 12-14 day culture by flow cytometric analysis of cells stained with FITC-anti-CD41a.

Flow cytometric analysis was performed using a Coulter Cytometry XL flow cytometer (Coulter; Hialeah, FL). Fluorescence attributable to FITC- and PE-labeled antibodies was excited by an argon laser operating at 488 nm. Emission from fluorescein and PE was measured using bandpass filters of 525 nm and 585 nm, respectively. The percentage of positive cells was corrected by subtracting the percentage of positive cells in the isotope-stained control within the same integration region.

Ploidy was determined by a modification of a procedure described by *Tomer et al.* [13]. Essentially, cells were labeled with FITC-anti-CD41a then fixed in 0.5% paraformaldehyde. DNA was then stained with propidium iodide in the presence of Triton X-100 for cell permeabilization. This was followed by RNA digestion. Samples were analyzed gating on peak versus integral red fluorescence (488 nm laser blocking filter, 600 nm dichroic, 635 nm bandpass filter) to exclude doublets. Green fluorescence was defined by a 525 nm bandpass filter. Ploidy was expressed by the ploidy index, defined as %4N + %8N + %16N + %32N/%2N.

In some experiments, aliquots of cultured cells were cytospun onto slides, fixed with methanol and stained with a mixture of anti-Ib and anti-IIb (Immunotech-Amac). Antibody binding was revealed with rhodamine-conjugated anti-mouse IgG $F(ab)'_2$ fragments (Immunotech-Amac).

Electron Microscopy

MK suspensions, washed with MK medium, were fixed in 3% glutaraldehyde in White's buffer [21]. Following washing in phosphate buffer, pellets were suspended in 1% osmium tetroxide in phosphate buffer for 30 min at 0°C. The samples were then dehydrated in a graded series of ethanol concentrations, then treated with propylene oxide and embedded in Epon 812. The sections were stained with uranyl acetate and lead citrate to enhance contrast. Examination was carried out in a Philips (Mahxah, NJ) 301 electron microscope.

Statistical Analysis

The results are expressed as the mean ± SE. Differences were evaluated using the Student's paired *t*-test, and p values <0.05 were considered statistically significant.

Results

Determination of MK Progenitors in Purified $CD34^+$ Cells

A recovery of about 60% $CD34^+$ cells was obtained with a purity of 86.4% ± 1.5 SE ($n = 6$) on the basis of flow cytometric analysis following staining with PE-anti-CD34 (HPCA-2). The relative frequency of MK progenitors staining with both anti-CD34 and anti-CD41a was 5.8% ± 0.4 SE ($n = 6$) (Fig. 1). In Figure 1, a representative flow cytometric pattern showed co-expression of CD34 and CD41a antigens in 4.3% of cells.

Effect of TPO, IL-3 or SCF on Cultures of MNC and Purified $CD34^+$ Cells

In three different experiments no MKs were obtained when MNC were cultured in serum-free media in the presence of TPO alone or TPO in combination with IL-3 or SCF. The addition of serum or heparinized platelet-poor plasma to TPO-treated MNC cultures resulted in significant MK production. Therefore, all the experiments were carried out in the presence of 2.5% normal serum and 50 U/ml of TPO which were found to be optimal for MK growth. Culture of MNC under these conditions produced MKs which exhibited a normal morphology (Fig. 2). A small proportion of MKs produced platelet-like elements (Fig. 3).

In 12-day cultures, Figure 4 shows that while TPO induced MK proliferation in a

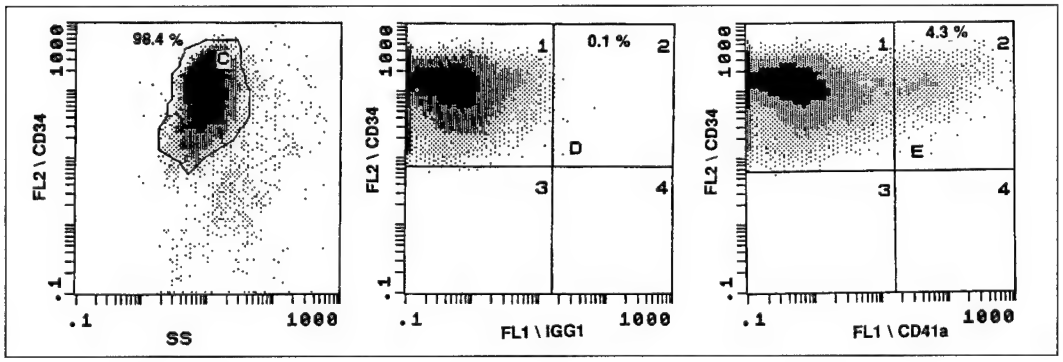

Fig. 1. Representative flow cytometric analysis of coexpression of CD34 and CD41a in purified CD34⁺ cells. Left, *CD34-PE versus side scatter (SS) of the cell population, over 90% of the gated cells express CD34⁺;* Middle, *CD34-PE versus isotypic control;* Right, *two-color analysis of the gated CD34⁺ population (CD34-PE versus CD41a-FITC) showing 4.3% double-stained cells.*

concentration-dependent manner, MK ploidy was inversely related to TPO concentration. In five separate experiments, increasing the culture time to 17 and 20 days did not significantly change the proliferation and ploidy patterns induced by TPO (data not shown).

Figure 5 shows the effect of TPO, IL-3, and SCF on MK proliferation in cultures of MNC and CD34⁺ cells. TPO alone, TPO + IL-3, and TPO + SCF stimulated greater numbers of MKs per seeded CD34 cell in MNC cultures compared to CD34⁺ cultures (TPO alone, $p = 0.03$; TPO + IL-3, $p = 0.01$; TPO + SCF, $p = 0.01$), except when TPO was used in combinations with both IL-3 and SCF. IL-3 at a concentration of 0.16 ng/ml potentiated the effect of TPO on MK growth from either MNC or CD34⁺ cell cultures ($p < 0.005$ for both MNC and CD34⁺, $n = 7$). The IL-3 effect was more pronounced with purified CD34⁺ cells (a three-fold increase in proliferation) than with MNC (a 1.4-fold increase). The absolute numbers of MKs obtained per seeded CD34⁺ cell in CD34⁺ cultures were 0.02 ± 0.002 (mean ± SE), 1.14 ± 0.21, and 3.86 ± 0.78 in the presence of IL-3, TPO, and IL-3 + TPO, respectively ($p < 0.05$, $n = 4$). These results indicate synergism between IL-3 and TPO. The relative frequency of MKs was 32.1 ± 3 SE and 55.8 ± 3 SE ($n = 11$) in TPO-stimulated cultures of MNC and CD34⁺ cells, respectively, and was not affected by IL-3. In two separate experiments, MNC were cultured in the presence of TPO and increasing concentrations of IL-3. Representative numbers of MKs obtained per seeded CD34⁺ cells were 4, 11.5 and 10 when cultures were performed using 0.16, 1.6 and 16 ng/ml, respectively, indicating that 1.6 ng/ml of IL-3 provides maximal MK proliferation.

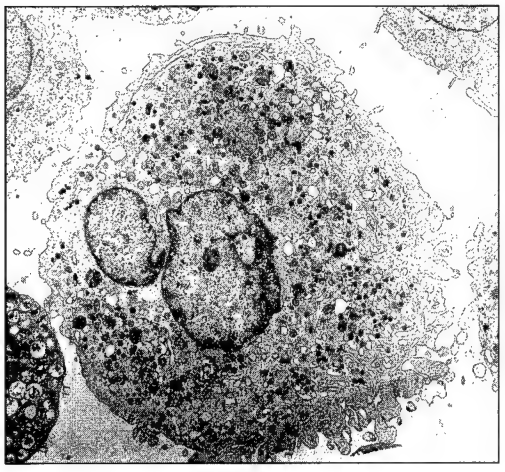

Fig. 2. Ultrastructure of bone marrow megakaryocytes cultured with TPO. *Normal distribution of α-granules and demarcation membranes is observed. Magnified ×640.*

The addition of SCF to TPO did not have any effect on MK growth in cultures of either MNC or purified CD34⁺ cells (Fig. 5). While IL-3 potentiated the effect of TPO in purified

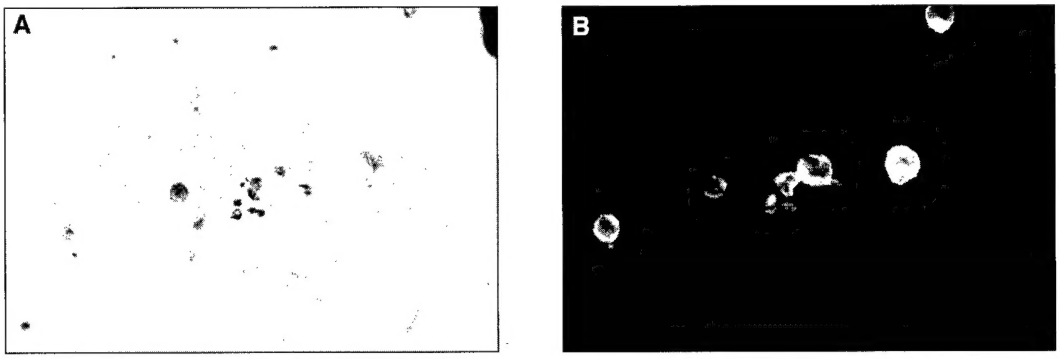

Fig. 3. A) Phase contrast microscopy of cultured megakaryocytes showing production of platelet-like elements; B) same field after staining with a mixture of anti-Ib and anti-IIb/rhodamine.

$CD34^+$ cells when added in combination with SCF ($p < 0.005$, $n = 7$), IL-3 did not enhance TPO activity when added with SCF in MNC.

The potentiating effect of IL-3 and SCF on TPO-stimulated nuclear maturation of MKs was analyzed by ploidy measurements. IL-3 and SCF did not alter TPO-stimulated ploidy of MNC or $CD34^+$ cells. On the other hand, a statistically significant higher ploidy was obtained in MNC than in purified $CD34^+$ cells when IL-3 was used in combination with either TPO or TPO and SCF (Fig. 6).

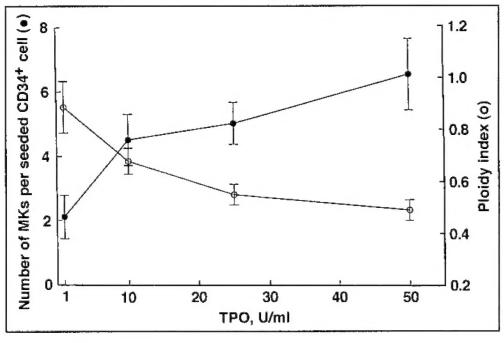

Fig. 4. Effect of different TPO concentrations on MK proliferation and ploidy in MNC cultures. *(●) proliferation expressed by absolute number of MKs/seeded $CD34^+$ cells; (○) ploidy index (%4N + %8N + %16N + %32N/%2N). Inverse relationship is observed between proliferation and ploidy. Each value represents the mean ± SE of seven to nine separate experiments.* $p < 0.005$ *for 1 and 10 U/ml of TPO versus 50 U/ml TPO for both MK/CD34 and ploidy.*

Effect of BM Stroma on TPO-Stimulated Cultures of Purified $CD34^+$ Cells

In order to evaluate the contribution of human BM stroma to the TPO effect on megakaryocytopoiesis, we cultured $CD34^+$ cells either in direct contact with stroma or in inserts separated from stroma by a semipermeable membrane (noncontact). Under "noncontact" conditions, $CD34^+$ cells yielded significantly more MKs in the presence of human stroma than in its absence (stroma-free) (Fig. 7). The potentiating effect of stroma was only observed in cell proliferation and not in the relative frequency of MKs. The smaller number of MKs obtained in "contact" conditions was due to the adherence of MKs to stromal cells. When similar experiments were performed using the

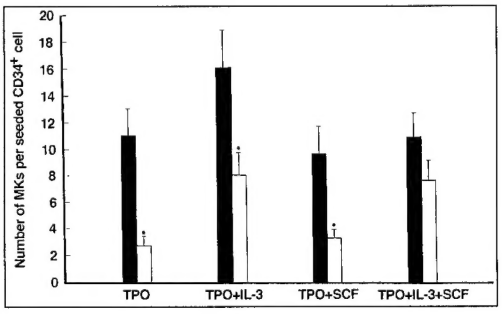

Fig. 5. Effect of TPO, IL-3 and SCF on MK proliferation in cultures of MNC and $CD34^+$ cells. *Black bars, MNC; Empty bars, $CD34^+$ cells. Each value represents the mean ± SE of seven separate experiments.* $^*p < 0.05$ *when comparing MNC with $CD34^+$ cells.*

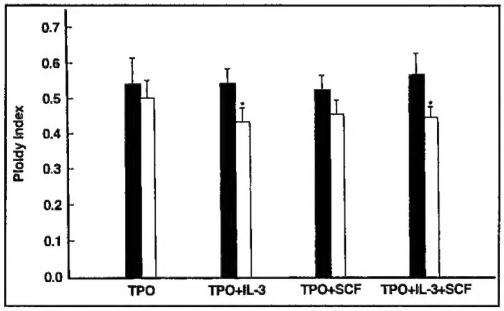

Fig. 6. Effect of TPO, IL-3 and SCF on MK ploidy in cultures of MNC and CD34⁺ cells. *Black bars, MNC; Empty bars, CD34⁺ cells. Mean (± SE) number of ploidy index (%4N + %8N + %16N + %32N/%2N). Each value represents the mean ± SE of eight separate experiments.* *$p < 0.05$ *when comparing MNC with CD34⁺ cells.*

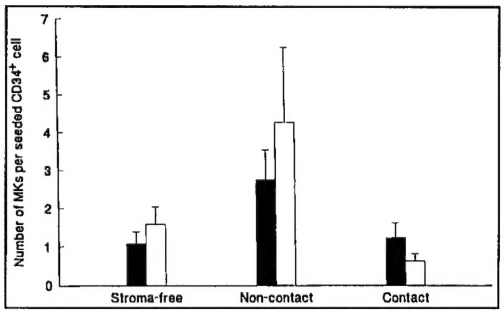

Fig. 7. Effect of human and murine stroma on TPO-stimulated CD34⁺ cells. *Black bars, human stroma; Empty bars, M2-10B4 murine stroma cell line. Mean (± SE) number of CD41a⁺ cells per seeded CD34⁺ cell by study condition. For human stroma,* $p = 0.04$ *for "stroma-free" versus "noncontact."*

M2-10B4 murine stroma cell line, the difference between noncontact and stroma-free conditions was not statistically significant. As in human stroma, the low values of MKs obtained in the contact system were related to the adherence of MKs to the stromal layer.

Discussion

Most studies on the effect of TPO on megakaryocytopoiesis have been carried out using clonogenic assays in murine systems [9, 10]. In this study, we evaluated the effect of TPO in human bone marrow liquid culture systems for the purpose of designing a protocol for ex vivo expansion of megakaryocytes. In the murine system, MK proliferation and ploidy increase with TPO concentration [10], while in the human BM system, an inverse relationship was observed between proliferation and ploidy. Since ploidy did not increase in cultures extended to 12, 17 or 20 days, it is unlikely that the low ploidy values are related to continuing cell proliferation. Our results are in agreement with previous data showing that in IL-3- or SCF-treated cultures, MK endoreplication proceeds synchronously with replication and is completed before the MKs exhaust their replicative potential. Unlike the reported presence of autophagic particles in murine MKs cultured with TPO [22], the cultured human MKs exhibited a normal ultrastructure and produced platelet-like elements.

The effect of IL-3 and SCF on TPO-stimulated human megakaryocytopoiesis in BM cultures of MNC and CD34⁺ cells was different than in the murine system [9, 10]. In the human system, while IL-3 synergized with TPO to stimulate megakaryocytopoiesis in cultures of both MNC and purified CD34⁺ cells, the effect of IL-3 was more potent in purified CD34⁺ cells than in MNC. Higher numbers of MKs per seeded CD34⁺ cells in cultured MNC compared to cultured CD34⁺ cells were obtained with TPO alone or supplemented with IL-3 or SCF but not when TPO was added with both IL-3 and SCF. Since MNC and purified CD34⁺ cells were seeded at equivalent concentrations of CD34⁺ cells, the enhancing effect of MNC could be due to the contribution of additional growth factors released by accessory cells. The relative frequency of MKs in TPO-containing cultures was not affected by IL-3. Therefore, the increase of the absolute number of MKs per seeded CD34⁺ cells in the presence of IL-3 was due to a general proliferation of all cell types. No effect of SCF was observed when used in combination with TPO in cultures of either MNC or purified CD34⁺ cells. A possible explanation is that TPO targets the same population of early progenitors as SCF. In this respect, *Methia et al.* [23] reported the expression of *c-mpl* receptor mRNA in the early CD34⁺/CD38⁻ stem cell population. When SCF was used in combination with TPO and IL-3 in MNC cultures, the potentiating effect of IL-3 on TPO-stimulated megakaryocytopoiesis was abolished. This effect, which was not observed in cultures of purified CD34⁺ cells, may be due to an as yet

undetermined action of SCF on accessory cells present in the MNC population. In the murine system, unlike the human system, SCF synergized with TPO to stimulate proliferation of early and late MK progenitors whereas IL-3 and TPO had additive and synergistic effects when tested on late and early MK progenitors, respectively [9, 10].

The effect of IL-3 and SCF on TPO-stimulated MK differentiation has not been reported in the murine system. In human BM, IL-3 used in combination with TPO, in the presence or absence of SCF, induced a higher ploidy in MNC than in purified $CD34^+$ cells. This points to a potentiating activity of accessory cells not only on MK proliferation but also on their differentiation.

The effect of marrow environment on megakaryocytopoiesis has been recently reported. Although macrophages exhibited an overall stimulatory effect on megakaryocytopoiesis due to their secretion of numerous cytokines [24], they inhibited colony-forming unit-MK growth in amegakaryocytic thrombocytopenia [25]. While unactivated human BM microvascular endothelial cells induced adherence of human $CD34^+$ cells and MKs [26], activated human umbilical vein endothelial cells induced adherence of human MKs and increased MK maturation without affecting proliferation [11]. On the other hand, human fibroblasts induce MK proliferation without affecting their maturation [12]. In our study, the overall effect of marrow environment from human stroma on cultures of purified $CD34^+$ cells pointed to a synergistic proliferative effect with TPO due to the release of soluble factor(s). When $CD34^+$ cells were cocultured in physical contact with human BM stroma a large proportion of MKs adhered to stroma cells. The effect of stroma on proliferation and differentiation of these adherent cells is yet to be determined. As another possible source of stroma we evaluated the effect of a murine stromal cell line. Although four out of five cultures of $CD34^+$ cells elicited more MKs in the "noncontact" as compared to the "stroma-free" conditions, no statistical difference in MK growth was found upon comparing the two conditions. This could be due to the great variability found from one marrow sample to another.

In conclusion, our studies show that a different pattern emerges when comparing murine and human megakaryocytopoiesis. This may be related to different frequencies of progenitor populations as well as different architecture of cytokine receptors. Due to the presence of accessory cells, MNC appear more efficient in producing MKs than purified $CD34^+$ cells. Nonetheless, purified $CD34^+$ cells should be considered for ex vivo expansion in breast cancer or neuroblastoma clinical trials since malignant cells in these disorders may be readily separated from $CD34^+$ cells [27-29]. The potentiating effect of IL-3 on MK progenitors is of particular interest in view of its effect in supporting a basal level of megakaryocytopoiesis in the absence of TPO [22]. Furthermore, the efficiency of $CD34^+$ cells in producing MKs might be enhanced with the use of conditioned medium obtained from autologous irradiated stroma. In summary, the results reported herein should facilitate the development of ex vivo MK expansion for providing platelet support for transplant and chemotherapy-related thrombocytopenic patients.

Acknowledgments

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